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Genetic approaches in mice to understand Rel/NF-κB and IκB function: transgenics and knockouts

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Rel/NF-κB transcription factors have been implicated in regulating a wide variety of genes important in cellular processes that include cell division, cell survival, differentiation and immunity. Here genetic models in which various Rel/NF-κB and IκB proteins have either been over-expressed or deleted in mice will be reviewed. Although expressed fairly ubiquitously, homozygous disruption of individual Rel/NF-κB genes generally affects the development of proper immune cell function. One exception is *rela*, which is essential for embryonic liver development. The disruption of genes encoding the individual subunits of the IκB kinase, namely IKKα and IKKβ, has demonstrated that IKKβ transmits the response to most common NF-κB inducing agents, whereas IKKα has an unexpected role in keratinocyte differentiation. Future studies will no doubt focus on the effect of multiple gene disruptions of members of this signaling pathway, on tissue-specific disruptions of these genes, and on the use of these mice as models for human diseases.

Keywords: NF-κB; Rel; IκB; IKK; knockout mice; transgenic mice; mouse genetics

Introduction

In mammals, there are five distinct Rel/NF-κB transcription factor subunits–p50/p105, p52/p100, c-Rel, RelA and RelB–each encoded by a unique gene. Two genes, *nfkb1* and *nfkb2*, encode large cytoplasmic proteins (p105 and p100, respectively) with inhibitor IκB properties, and smaller DNA-binding subunits (p50 and p52, respectively) that correspond to the conserved N-terminal domain shared by all Rel/NF-κB proteins. The proteins encoded by c-*rel*, *rela* and *relb* contain C-terminal transcriptional transactivation domains in addition to their Rel Homology (RH) domains. Rel/NF-κB proteins bind to specific DNA target sites (κB sites) as heterodimers or homodimers. The most common complex in many cells is the p50-RelA heterodimer, usually specifically referred to as NF-κB. Rel/NF-κB dimers usually do not promote transcription if they lack a subunit with a C-terminal transactivation domain.

In most cell types, Rel/NF-κB transcription complexes are present as latent, cytoplasmic forms, which can be induced to enter the nucleus and activate gene expression. The cytoplasmic sequestration of Rel/NF-κB is regulated by the family of IκB inhibitor proteins that includes IκBα, IκBβ, IκBγ, IκBε and Bcl-3. Two major kinases, IKKα and IKKβ are responsible for the induced phosphorylation of IκBα and IκBβ, and this phosphorylation targets for IκBs for proteasome-dependent degradation and thus releases the Rel/NF-κB complex. The generation of transgenic mice over-expressing exogenous copies of particular Rel/NF-κB or IκB genes, and of mice homozygous for null mutations in Rel/NF-κB, IκB or IKK genes, has provided invaluable models for elucidating the physiological functions and regulation of the components in the Rel/NF-κB signal transduction pathway. The phenotypes of the various transgenic and mutant mouse models are summarized herein.

Null mutations for Rel/NF-κB proteins

Single mutations

nfkb1$^{-/-}$ *mice* *nfkb1* encodes primarily two proteins, a 105 kDa non-DNA binding cytoplasmic molecule (p105) and a 50 kDa DNA-binding protein (p50) that corresponds to the N terminus of p105. In addition, in certain mouse cells, an *nfkb1*-encoded protein (IκBγ) containing only the C-terminal half of p105 has been detected (Inoue *et al.*, 1992). Despite the nearly ubiquitous expression of *nfkb1* and the role of p50 as the major partner of RelA, which is required for normal embryogenesis (see below), mice lacking p50 and p105 (*nfkb1*$^{-/-}$ mice) develop normally and exhibit no histopathological changes. Although p50/p105 is not essential for hemopoiesis, *nfkb1*$^{-/-}$ mice exhibit multiple defects in the function of the immune system (Sha *et al.*, 1995). Mature quiescent *nfkb1*$^{-/-}$ B cells turn over more rapidly *in vivo* and undergo accelerated apoptosis in culture (Grumont *et al.*, 1998), indicating that p50/p105 is required for the survival of non-activated B cells. When activated with lipopolysaccharide and soluble CD40 ligand, *nfkb1*$^{-/-}$ splenic B cells proliferate poorly. However, these cells respond normally to antigen receptor ligation and anti-IgD dextran antibodies (Sha *et al.*, 1995; Snapper *et al.*, 1996), indicating that p50/p105 is only essential for certain B-cell activation pathways. The B-cell proliferative defects in *nfkb1*$^{-/-}$ mice are due to a cell-cycle block in G1 and enhanced mitogen-induced apoptosis (Grumont *et al.*, 1998).

Consistent with a role for *nfkb1* in B-cell activation, *nfkb1*$^{-/-}$ mice fail to mount a normal humoral

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response when challenged with various antigens (Sha *et al*., 1995). This is due, in part, to cell autonomous defects in heavy chain isotype switching resulting from impaired transcriptional induction of germline C_H genes by mitogens and cytokines (Snapper *et al*., 1996), the expression of which is required for immunoglobulin gene rearrangement. Interestingly, the susceptibility of *nfkb1*$^{-/-}$ mice to various pathogens varies quite markedly. These mice are more susceptible to *Listeria monocytogenes* and *Streptococcus pneumoniae*, respond normally to *Haemophilus influenza* and *Escherichia coli*, and are more resistant to murine encephalomyocarditis (EMC) virus (Sha *et al*., 1995). Enhanced resistance to EMC correlates with increased production of β-interferon by *nfkb1*$^{-/-}$ cells (Sha *et al*., 1995), thereby implicating p50/p105 as a regulator of innate as well adaptive immune responses.

nfkb2$^{-/-}$ *mice* Similar to *nfkb1*, the *nfkb2* gene encodes a 52 kDa protein that corresponds to the N-terminus of a larger 100 kDa protein. However, unlike *nfkb1*, *nfkb2* expression is restricted to the epithelium of the stomach and select areas of hemopoietic organs such as the thymic medulla, the marginal zone and periarterial sheath of the spleen (Attar *et al*., 1997). Mice lacking p52/p100 proteins develop normally, with the major defect being a disruption of splenic and lymph node architecture (Caamaño *et al*., 1998; Franzoso *et al*., 1998). In the spleen of these mice, the perifollicular marginal zone, thought to be important for regulating cell migration during immune responses, is absent and the B-cell follicular areas are either absent or depleted. Although the failure of these mice to mount a normal T cell-dependent antibody response is associated with an inability to form germinal centers, this cannot simply be explained by intrinsic B- or T-cell defects, as *nfkb2*$^{-/-}$ lymphocytes exhibit only mildly impaired proliferative responses coupled with normal antibody or cytokine production when activation in culture (Franzoso *et al*., 1998). Instead, these immune deficiencies appear to reflect a defect in antigen presentation by accessory cells. A role for p52/p100 in the regulation of antigen presentation is consistent with the findings that the hemopoietic restricted sites of *nfkb2* expression contain macrophages and dendritic cells and that p52 is a major dimer partner of RelB, another Rel/NF-κB family member important for the function and development of dendritic cells.

c-rel$^{-/-}$ *mice* Restricted expression of c-Rel to lymphocytes, monocytic, granulocytic, and erythroid cells in mouse fetal and adult hemopoietic organs coincides with the development and expansion of the hemopoietic system. Consistent with this pattern of expression, c-Rel is essential for a variety of functions in hemopoietic cells, although it is dispensable for mouse embryonic development. Although normal numbers of hemopoietic cells in c-*rel*$^{-/-}$ mice indicate that c-Rel is not essential for the differentiation of hemopoietic precursors, mature lymphocytes and macrophages exhibit a number of activation-associated defects associated with B- and T-cell proliferation, isotype switching and the production of various cytokines and immune modulatory molecules (Gerondakis *et al*., 1996; Grigoriadis *et al*., 1996; Grumont *et al*., 1998; Köntgen *et al*., 1995).

Impaired c-*rel*$^{-/-}$ B-cell proliferation in response to range of individual mitogens is due to a cell-cycle block in G1 and elevated activation-induced apoptosis (Grumont *et al*., 1998). While c-Rel-regulated gene(s) critical for cell-cycle progression remained to be determined, mitogen-induced apoptosis in c-*rel*$^{-/-}$ B cells is due in part to a failure to upregulate the expression of *A1*, a Bcl-2 prosurvival homologue directly regulated by c-Rel (Grumont *et al*., 1999). In c-*rel*$^{-/-}$ B cells, defects in immunoglobulin C_H switching implicate c-Rel in various steps of the switching process such as germline C_H gene transcription. In contrast to the cell autonomous defects that afflict c-*rel*$^{-/-}$ B-cell proliferation, the failure of c-*rel*$^{-/-}$ T cells to proliferate in response to mitogens can be overcome by exogenous interleukin-2 (IL-2) (Gerondakis *et al*., 1998). IL-2 is not, however, the only cytokine whose expression is impaired, as activated c-*rel*$^{-/-}$ T cells also express reduced amounts of IL-3 and GM–CSF (Gerondakis *et al*., 1996). c-Rel also serves distinct roles in different macrophage populations. Whereas GM-CSF, G-CSF, IL-6, TNFα and iNOS expression is abnormal in activated c-*rel*$^{-/-}$ resident peritoneal macrophages, only TNFα and IL-6 expression is impaired in stimulated c-*rel*$^{-/-}$ elicited peritoneal macrophages (Grigoriadis *et al*., 1996). An elevation of GM-CSF expression in certain c-*rel*$^{-/-}$ macrophage populations (Grigoriadis *et al*., 1996), but reduced GM–CSF expression in c-*rel*$^{-/-}$ T cells (Gerondakis *et al*., 1996) highlights the tissue-specific modulation of c-Rel function and establishes that mammalian c-Rel, like *Drosophila* Dorsal (see Govind, 1999), is both an activator and repressor of gene expression. *In vitro* defects in c-*rel*$^{-/-}$ lymphocyte and macrophate function are also reflected in the impaired innate and adaptive immune response of c-Rel-deficient mice (Gerondakis *et al*., 1996; Harling-McNabb *et al*., 1999; Köntgen *et al*., 1995).

rela$^{-/-}$ *mice* The absence of RelA leads to embryonic lethality between days E15 and E16 post-coitum, a result of fetal hepatocyte apoptosis (Beg and Baltimore, 1996). The death of *rela*$^{-/-}$ fetal hepatocytes arises from their heightened sensitivity to the cytotoxic effects of TNFα, as evidenced by the observation that an absence of this cytokine rescues *rela*$^{-/-}$ mice from embryonic lethality (Doi *et al*., 1999). Consistent with RelA playing an anti-apoptotic role in different cell types, *rela*$^{-/-}$ fibroblasts and macrophages also exhibit increased sensitivity to apoptosis induced by TNFα (Beg and Baltimore, 1996). Although several Rel/NF-κB regulated prosurvival genes including *A1* (Zong *et al*., 1999), *cIAP2* (Wang *et al*., 1998) and *IEX-1L* (Wu *et al*., 1998) are normally upregulated by TNFα, it remains to be determined which, if any, of these is critical in protecting hepatocytes from TNFα-induced apoptosis (see also Barkett and Gilmore, 1999). RelA is also important for normal lymphocyte function. The analysis of SCID mice reconstituted with day E13 *rela*$^{-/-}$ fetal liver cells has established that RelA, while dispensable for lymphopoiesis, is required for mitogen-induced lymphocyte proliferation and isotype switching (Doi *et al*., 1997).

relb$^{-/-}$ *mice* RelB expression is normally confined to dendritic cells and B lymphocytes. Mice lacking RelB

exhibit multiple pathological lesions and defects in acquired and innate immunity. Pathological conditions that afflict *relb*$^{-/-}$ mice include T-cell inflammatory infiltrates of various organs, T cell-dependent myeloid hyperplasia and splenomegaly due to extramedullary hemopoiesis (Burkly *et al.*, 1995; Weih *et al.*, 1995). The basis of the inflammatory pathology in *relb*$^{-/-}$ mice remains unclear, but may be due to the absence of certain thymic and splenic dendritic cell (DC) populations (Wu *et al.*, 1998). Although the thymic DC deficiency in *relb*$^{-/-}$ mice is a secondary consequence of the disrupted thymic architecture, the absence of splenic myeloid related CD8α DCs is a direct stem cell intrinsic defect (Wu *et al.*, 1998). This deficit of thymic and splenic DCs may account for the inability of *relb*$^{-/-}$ mice to effectively delete autoreactive thymocytes and T cells (Burkly *et al.*, 1995), which in turn creates a pool of self-reactive T cells that ultimately give rise to the inflammatory phenotype. Delayed-type hypersensitivity and CD8$^+$ cytotoxicity, macrophage-mediated immunity to various pathogens, and IgG responses to T cell-dependent antigens are also impaired (Weih *et al.*, 1997; Caamaño *et al.*, 1999). Because delayed-type hypersensitivity responses are dependent on Langerhans cells, this may indicate that these antigen-presenting cells are also defective in *relb*$^{-/-}$ mice. The findings that the proliferative response of *relb*$^{-/-}$ splenic B cells to mitogens in tissue culture is only reduced slightly and that Ig secretion and isotype switching are normal indicate that the humoral immune defects are most likely a secondary consequence of impaired CD4$^+$ T-cell function arising from deficiencies in antigen presentation. These findings indicate that in contrast to the other Rel/NF-κB proteins, each of which is dispensable for normal hemopoiesis, RelB is required for the development of specific dendritic cell populations.

Multiple mutations

Despite the unique roles ascribed to individual Rel/NF-κB subunits, functional overlap and redundancy among these proteins most likely prevents the emergence of certain phenotypes in single mutant mice due to compensation by other family members. Indeed, mice lacking multiple Rel/NF-κB proteins often exhibit novel phenotypes or more severe versions of those phenotypes seen in single mutants.

nfbk1$^{-/-}$ nfkb2$^{-/-}$ *mice* Mice deficient in both *nfkb1* and *nfkb2*, while phenotypically indistinguishable from control litter mates at birth, soon exhibit growth retardation and cranofacial abnormalities, the latter being a result of bone thickening due to osteopetrosis (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997). Bone remodeling is dependent on bone resorption by myeloid lineage-derived osteoclasts. Although osteoclast numbers are markedly reduced in these double mutant mice and *nfkb1*$^{-/-}$ *nfkb2*$^{-/-}$ osteoclast progenitors cannot differentiate *in vitro* (Iotsova *et al.*, 1997), transplantation of normal marrow into newborn *nfkb1*$^{-/-}$ *nfkb2*$^{-/-}$ mice only partially rescues this osteopetrotic phenotype (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997). This indicates that the combined deficiency of *nfkb1* and *nfkb2* afflicts cells of a hemopoietic origin and the bone marrow microenvironment. Target gene(s) important for normal bone development that are affected by the absence of *nfkb1* and *nfkb2* remain to be identified.

In contrast to the single mutant mice, B-cell development is blocked in *nfkb1*$^{-/-}$ *nfkb2*$^{-/-}$ double-mutant mice at the immature IgM$^+$IgD$^-$ stage (Franzoso *et al.*, 1997), which normally corresponds to those cells newly emerged from the bone marrow. Collectively, these findings indicate that these two non-transactivating NF-κB proteins perform redundant functions in bone development and B-cell differentiation.

nfkb1$^{-/-}$ relb$^{-/-}$ *mice* The absence of *nfkb1*-encoded proteins exacerbates the severity and extent of organ inflammation resulting from an absence of RelB, with the mice dying within 3–4 weeks of birth (Weih *et al.*, 1997). While myeloid hyperplasia is more pronounced, the inflammatory infiltrates in *nfkb1*$^{-/-}$ *relb*$^{-/-}$ mice are devoid of B cells, the result of a B-cell developmental defect that leads to a marked reduction in both B220$^+$ splenic and bone marrow cells. This phenotype indicates that p50-containing complexes partly compensate for RelB function in dendritic cells and that p50/p105 and RelB perform redundant functions in B-cell development.

nfkb1$^{-/-}$ *c*-rel$^{-/-}$ *mice* Embryogenesis is normal in mice that lack p50 and c-Rel (Pohl *et al.*, in preparation). While both transcription factors are also dispensable for the differentiation of hemopoietic precursors, immune defects in these double mutants are more severe than in individual c-*rel*$^{-/-}$ or *nfkb1*$^{-/-}$ mice (Pohl *et al.*, in preparation). *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ lymphocytes fail to divide when stimulated with any combination of mitogens. Whereas *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ B cells cannot exit the G0 stage of the cell cycle when treated with mitogens, *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ T cells can undergo blast formation, indicating that the G0-G1 transition is differentially regulated by Rel/NF-κB in B and T cells. Consistent with the increased severity of the *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ lymphocyte activation defects, humoral immunity is further diminished in the double-mutant mice. This is due in part to the lack of germinal centers and an absence of germline C_H gene expression. This demonstrates that functional redundancy of c-Rel and p50 dimers is only important in the immune system.

rela$^{-/-}$ *c*-rel$^{-/-}$ *mice* *rela*$^{-/-}$ c-*rel*$^{-/-}$ double mutants, like *rela*$^{-/-}$ single mutant mice, die as a result of fetal hepatocyte apoptosis, with the onset of liver degeneration occurring 1–1.5 days earlier in gestation (E13–E13.5) than in *rela*$^{-/-}$ single mutants (Grossmann *et al.*, 1999) This indicates that c-Rel partly compensates for the anti-apoptotic function of RelA, which is consistent with the anti-apoptotic effects of c-Rel and RelA *in vitro* (see Barkett and Gilmore, 1999). The combined loss of c-Rel and RelA is also associated with multiple hemopoietic cell defects (Grossmann *et al.*, 1999). Lethally-irradiated mice engrafted with E12 *rela*$^{-/-}$ c-*rel*$^{-/-}$ fetal liver hemopoietic precursors die from the combined effects of anemia and granulocytosis. The anemia in reconstituted mice appears to reflect a defect in erythrocyte differentiation rather than a reduction in erythroid progenitors, as the

number of erythroid colonies in cultures of double mutant fetal liver cells is normal. Consistent with a developmental defect in erythropoiesis, the fetal blood of E13 *rela*$^{-/-}$ c-*rel*$^{-/-}$ embryos has higher than normal numbers of nucleated embryonic erythrocytes. This persistence of embryonic erythrocytes in *rela*$^{-/-}$ c-*rel*$^{-/-}$ embryos suggests that the switch from primitive to definitive erythropoiesis is impaired.

Monocyte differentiation is also affected in these mice. The loss of both c-Rel and RelA results in cell death during monocyte differentiation in culture. In contrast, c-*rel*$^{-/-}$ *rela*$^{-/-}$ monocytic precursors appear to differentiate normally *in vivo*, indicating that compensatory signals or factors critical for monocyte differentiation and survival that are missing *in vitro* operate *in vivo*.

Although the combined loss of c-Rel and RelA does not impair thymocyte development or B-cell differentiation in the bone marrow, *rag1*$^{-/-}$ mice reconstituted with double mutant fetal liver hemopoietic precursors exhibit a marked reduction in the number of peripheral B and T cells (Grossmann *et al.*, in preparation). *rela*$^{-/-}$ c-*rel*$^{-/-}$ B cells (IgM$^+$IgD$^-$) newly emerged from the bone marrow fail to mature to IgM$^-$IgD$^+$ cells, a finding consistent with very low serum immunoglobulin levels and an absence of B cells in the lymph nodes of these mice (Grossmann *et al.*, 1999). The death of double mutant *rela*$^{-/-}$ c-*rel*$^{-/-}$ IgM$^+$IgD$^+$ B cells is dramatically accelerated *in vitro* and these cells turnover more rapidly than their normal or single mutant counterparts *in vivo*. Consequently, the failure of *rela*$^{-/-}$ c-*rel*$^{-/-}$ B cells to enter the mature B-cell pool after exiting the bone marrow appears to be due to a reduced lifespan. In contrast, the profound reduction of mature CD4$^+$ and CD8$^+$ T cells in the spleen and lymph nodes of engrafted *rag1*$^{-/-}$ mice is not associated with increased cell death; instead it appears to be linked to a defect in the post-thymic expansion of *rela*$^{-/-}$ c-*rel*$^{-/-}$ T cells. This indicates that although c-Rel and RelA are essential for the generation of mature B and T cells, these transcription factors only appear to be important for survival in B-cells

nfkb1$^{-/-}$ rela$^{-/-}$ *mice* The absence of both p50 and RelA (the NF-κB complex), like the combined loss of c-Rel and RelA, leads to an earlier onset of embryonic death (around E13) due to fetal liver apoptosis (Horvitz *et al.*, 1997). Irradiated mice engrafted with E12 *nfkb1*$^{-/-}$ *rela*$^{-/-}$ fetal liver hemopoietic precursors lack B220$^+$ cells in the bone marrow, spleen and blood, indicating that the defect in B lymphopoiesis occurred at a stage before the development of B220$^+$ B-cell precursors (Horvitz *et al.*, 1997). Simultaneous transplantation of wild-type bone marrow cells rescues the production of *nfkb1*$^{-/-}$ *rela*$^{-/-}$ B cells, a finding consistent with NF-κB mediating the development or survival of an early lymphocyte precursor by regulating an extracellular factor. B cells lacking both p50/p105 and RelA exhibit profound proliferative defects in response to mitogen stimulation (Horvitz *et al.*, 1999). Mice engrafted with NF-κB deficient fetal liver cells also develop a fetal liver-derived granulocytosis (Horvitz *et al.*, 1997). Combined with the observation that mice receiving *rela*$^{-/-}$ c-*rel*$^{-/-}$, but not *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ fetal liver hemopoietic precursors develop granulocytosis, indicates that certain Rel/NF-κB proteins are important in regulating granulocyte homeostasis *in vivo*.

C-terminal truncation mutations in Rel/NF-κB proteins

nfkb1$^{\Delta CT/\Delta CT}$ *mice* Mice lacking the portion of the *nfkb1* coding region that encompasses the C-terminal regulatory domain of p105 show a more severe phenotype than mice homozygous for an *nfkb1* null mutation (Ishikawa *et al.*, 1998). These mice (*nfkb1*$^{\Delta CT/\Delta CT}$ mice) develop splenomegaly, enlarged lymph nodes and have lymphocytic infiltrations of the lung and liver. They also exhibit heightened susceptibility to various opportunistic pathogens. The changes associated with dysregulated lymphocyte function appear to result from an increase in B-cell numbers that coincide with the hyperproliferative responsiveness of these cells to mitogens. In contrast, T cells from *nfkb1*$^{\Delta CT/\Delta CT}$ mice exhibit a weak reduction in proliferative capacity in culture and produce lower amounts of various cytokines after activation. Consistent with the evidence that the ankyrin repeats in the C terminus of p105 regulate cellular levels of p50, gel shift analysis indicates that the nuclear levels of p50 homodimers are elevated in the tissues of *nfkb1*$^{\Delta CT/\Delta CT}$ mice. These findings, together with those for the *nfkb2*$^{\Delta CT/\Delta CT}$ mice, reinforce the notion that tight regulation of Rel/NF-κB expression is crucial for normal cellular functions.

nfkb2$^{\Delta CT/\Delta CT}$ *mice* *nfkb2*$^{\Delta CT\text{-}\Delta CT}$ mice, which express the 52 kDa form encoded by *nfkb2* but lack the 100 kDa protein due to disruption of the *nfkb2* C-terminal coding region, appear normal at birth but develop multiple pathologies post-natally (Ishikawa *et al.*, 1997). These pathologies include gastric hyperplasia of the epithelial layer of the antrum, hyperkeratosis in the heart, lymphocytic infiltration in the lamina propia and hemopoietic abnormalities such as spleen and thymic atrophy, enlarged lymph nodes, and granulocytosis. The presence of increased numbers of lymphocytes in various tissues and lymph nodes is consistent with *nfkb2*$^{\Delta CT/\Delta CT}$ T cells being hyperresponsive to activation in culture. Tissues from *nfkb2*$^{\Delta CT/\Delta CT}$ mice that overexpress p52-containing nuclear complexes also upregulate several genes known to be controlled by Rel/NF-κB, including those encoding TNFα, I-CAM1 and ELAM-1. These findings indicate that p52 is normally involved in controlling the growth of gastric mucosal cells and mature peripheral lymphocytes, and that dysregulated expression of this transcription factor can lead to hyperproliferation, a finding consistent with the rearrangement of *nfkb2* in certain human lymphomas (Rayet and Gélinas, 1999).

c-rel$^{\Delta CT/\Delta CT}$ *mice* Deletion of the c-Rel C-terminal transactivation domains creates a protein still capable of forming homodimers or heterodimers with other NF-κB subunits and binding DNA, but unable to regulate transcription in a normal manner. Several months after birth, mice homozygous for this mutation (c-*rel*$^{\Delta CT/\Delta CT}$) develop hypoplastic bone marrow, splenomegaly, enlarged lymph nodes, and lymphoid hyper-

plasia (Carrasco *et al.*, 1998). Prior to the onset of dysregulated lymphocyte expansion, young c-*rel*$^{\Delta CT/\Delta CT}$ mice exhibit defects in B-cell activation and antibody synthesis and have increased susceptibility to *L. monocytogenes*, probably a result of reduced nitric oxide and GM–CSF production by macrophages. The molecular basis for the difference in the phenotypes of c-*rel*$^{-/-}$ and c-*rel*$^{\Delta CT/\Delta CT}$ mice most likely reflects in part functional compensation for c-Rel by other family members in the c-*rel*$^{-/-}$ mice versus the abnormal transcriptional activity of NF-κB-like complexes containing the C terminally-truncated c-Rel protein.

Null mutations for IκB proteins

ikba$^{-/-}$ *mice* IκBα is the major ubiquitous cytoplasmic inhibitor that is critical for regulating the rapid transient nuclear induction of Rel/NF-κB. Although the embryonic development of mice lacking IκBα appears to be normal, *ikba*$^{-/-}$ mice die 7–10 days post-natally, afflicted by severe widespread inflammatory dermitis and granulocytosis (Beg *et al.*, 1995; Klement *et al.*, 1996). Coincident with this phenotype, the expression of certain proinflammatory cytokines and factors associated with granulocyte recruitment, adherence and activation such as TNFα, G-CSF, MIP-2 and VCAM-1 is increased. However, not all genes known to be induced by Rel/NF-κB are upregulated in *ikba*$^{-/-}$ cells, underscoring the role of other transcriptional regulators in the activation of many Rel/NF-κB target genes. Furthermore, despite the absence of IκBα in all tissues, changes in the constitutive nuclear levels of Rel/NF-κB are cell type-dependent. For example, whereas an increase in constitutively nuclear p50/RelA and p50 homodimers was observed in *ikba*$^{-/-}$ thymocytes and splenocytes, the levels of constitutive Rel/NF-κB complexes were unchanged in *ikba*$^{-/-}$ embryonic fibroblasts. This finding indicates that IκBα is more important in regulating the cytoplasmic retention of Rel/NF-κB in hemopoietic than certain non-hemopoietic cells. However, in response to activation signals, the nuclear localization of Rel/NF-κB in *ikba*$^{-/-}$ fibroblasts is prolonged, indicating that IκBα is essential for post-induction repression of Rel/NF-κB.

In the absence of p50/p105, most *nfkb1*$^{-/-}$ *ikba*$^{-/-}$ mice survive significantly longer (3–4 weeks) before succumbing to the same inflammatory disease as *ikba*$^{-/-}$ mice (Beg *et al.*, 1995). The absence of p50 significantly reduced constitutive nuclear levels of Rel/NF-κB in thymocytes, suggesting that the constitive expression of Rel/NF-κB may be critical in the neonatal lethality of *ikba*$^{-/-}$ mice.

To assess the extent of functional redundancy between IκBα and IκBβ, two IκB proteins that share extensive structural and biochemical similarities but different patterns of expression, a 'knock-in' strategy was employed. This involved deleting the *ikba* coding region and replacing it with the *ikbb* gene, which was now under the transcriptional control of *ikba* regulatory sequences. In contrast to *ikba*$^{-/-}$ mice, these homozygous knock-in mice are normal (Cheng *et al.*, 1998). Consistent with the absence of inflammatory disease, the regulation of Rel/NF-κB is equivalent to that of wild-type mice. This indicates that IκBα and IκBβ have similar biochemical properties and that these two inhibitors have acquired different functions primarily through a differential pattern of expression.

bcl-3$^{-/-}$ *mice* Bcl-3, a distinct member of the IκB-like protein family expressed primarily in hemopoietic tissue and liver, selectively inhibits DNA binding by p50 homodimers, but can also transactivate κB-dependent gene expression in the presence of p52 homodimers (Bours *et al.*, 1993). *bcl-3*$^{-/-}$ mice develop normally, but exhibit defects in antigen-specific B- and T-cell responses when challenged with various pathogens (Franzoso *et al.*, 1997; Schwartz *et al.*, 1997). Both the Th1 response to *T. gondii* and the capacity to produce specific high affinity T cell-dependent IgG2a antibodies to influenza are impaired in *bcl-3* knockout mice. Moreover, consistent with the failure to mount a normal antibody response, follicular splenic B-cell numbers are reduced and germinal centre formation is severely diminished. The underlying basis of these defects may be due in part to impaired antigen-dependent priming, since naive T *bcl-3*$^{-/-}$ cells polyclonally activated *in vitro* are able to produce normal levels of Th1 cytokines such as IFN-γ (Franzoso *et al.*, 1997). Such a defect is consistent with the depletion and loss of specific splenic marginal zone macrophage populations associated with the disrupted splenic architecture observed in *bcl-3*$^{-/-}$ mice.

ikbe$^{-/-}$ *mice* IκBε is a highly-specialized inhibitor of Rel/NF-κB complexes and is expressed at high levels primarily in T cells of the thymus and spleen, and to a lesser extent, in lung, ovary and testis (Li and Nabel, 1997; Mémet *et al.*, 1999). However, IκBε-deficient mice are viable, fertile, and lacking in severe immune defects (Mémet *et al.*, 1999). The only detected alterations in *ikke*$^{-/-}$ mice are a 50% reduction in the number of CDD44^{-}CD25^{+} T cells and increased expression of certain immunoglobulin isotypes and some cytokines. Nevertheless, *ikke*$^{-/-}$ mice show a normal response to several pathogens (Mémet *et al.*, 1999). In part, the minimal effect of loss of IκBε may be due to compensatory up-regulation of IκBα and IκBβ expression in *ikke*$^{-/-}$ mice, and *ikke*$^{-/-}$ *ikka*$^{-/-}$ double mutant mice die earlier post-natally than single *ikka*$^{-/-}$ mice (Mémet and Israël, personal communication).

Null mutations for the IκB kinases

The principal IκB kinase (IKK) is a complex containing two related catalytic kinases, IKKα and IKKβ, and the regulatory protein IKKγ, which is involved in kinase activation (Mercurio and Manning, 1999). Despite the sequence similarity of IKKα and IKKβ, the analysis of single knockout mice for these kinases has established that Rel/NF-κB activation by proinflammatory cytokines is dependent largely on IKKβ, whereas IKKα induces Rel/NF-κB during skin and skeletal development in response to an unidentified morphogenetic signal(s).

ikka$^{-/-}$ *mice* Mice homozygous for an *ikka* null mutation die post-natally, afflicted with multiple morphological defects, the most striking of which is the encasement of the embryo in a shiny taut skin that prevents the emergence of fore- and hind-limbs (Hu *et*

al., 1999; Takeda *et al.*, 1999). Other defects include an absence of ears, truncation of the head and snout, and skeletal abnormalities affecting the vertebrae, sternum, skull and digital phalanges that arise from the absence or inappropriate fusion of bones. Although the limbs of *ikka*$^{-/-}$ mice appears as ill-formed protrusions, beneath the skin they are almost normal in size, although lacking defined digits. The failure of limbs to emerge during embryogenesis appears to be the result of a block in keratinocyte differentiation, while the lack of distinct digits is due to an absence of programmed cell death that normally occurs within the interdigital regions.

The finding that loss of IKKα in mice causes developmental defects, suggests that, as in flies (Govind, 1999), Rel/NF-κB proteins regulate genetic programs in vertebrates that are associated with development as well as with immunity. This conclusion, while seemingly at odds with an absence of developmental defects in mice lacking individual Rel/NF-κB subunits, may emphasize the redundant function of Rel/NF-κB proteins in mammals. Alternatively and certainly possible, the IKK complex, especially IKKα, phosphorylates substrates other than IκB or regulates additional signaling pathways. Nevertheless, evidence of a role for Rel/NF-κB factors in vertebrate limb and skin development has previously come from over-expressing mutant IκBα in chick embryos (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998) and in the dermis of transgenic mice (Seitz *et al.*, 1998). The normal induction of Rel/NF-κB in *ikba*$^{-/-}$ embryonic fibroblasts in response to the pro-inflammatory cytokines TNFα or IL-1 occurs via IKKβ (Hu *et al.*, 1999; Takeda *et al.*, 1999), and this finding suggests that an unknown set of signals operating through IKKα is required for the induction of Rel/NF-κB during skin and skeletal development.

The Rel/NF-κB-regulated genes required for bone and skin differentiation that are activated via IKKα remain to be identified. Conservation of the vertebrate and invertebrate Rel/NF-κB pathways makes it highly likely that certain of the Dorsal-regulated genes important for embryonic pattern formation in flies will also be regulated by Rel/NF-κB in mammals. One such gene may be *twist*, the expression of which is reduced in *ikka*$^{-/-}$ embryos (Takeda *et al.*, 1999). The skull and bone defects in *ikka*$^{-/-}$ mice resemble the phenotypes seen in mice heterozygous for a null allele of *twist* and in people suffering from Saethre-Chotzen syndrome, an autosomal dominant disorder arising from mutations in *twist*. (Howard *et al.*, 1997). Other *ikka*$^{-/-}$ defects such as the lack of external ears, a partially split sternum and forked xiphoid resemble defects seen in mutations of various bone morphogenetic proteins (Hu *et al.*, 1999), suggesting that IKKα-dependent signals may regulate the localized expression of bone morphogenetic proteins.

ikkb$^{-/-}$ *mice* The loss of IKKβ leads to embryonic death between days E12.5 and E14.5 post-coitum (Li *et al.*, 1999; Tanaka *et al.*, 1999) and like *rela*$^{-/-}$ embryos, appears to result from fetal hepatocyte apoptosis. The conclusion that *ikkb*$^{-/-}$ and *rela*$^{-/-}$ mice die from a common defect is supported by the ability of TNF receptor I (Li *et al.*, 1999) and TNFα (Doi *et al.*, 1999) null mutants, respectively, to block the embryonic lethality associated with the loss of IKKβ and RelA. Consistent with a perturbation of TNFα signals leading to the death of *ikkb*$^{-/-}$ embryos, a weak induction of Rel/NF-κB in *ikkb*$^{-/-}$ mouse embryonic fibroblasts by TNFα establishes that proinflammatory cytokines induce Rel/NK-κB through IKKβ and not IKKα (Li *et al.*, 1999; Tanaka *et al.*, 1999). While the analysis of *in vitro* hemopoictic colony assays established from the fetal liver of *ikkb*$^{-/-}$ embryos indicates that hemopoiesis is normal in the absence IKKβ (Tanaka *et al.*, 1999), monocytic progenitors in the fetal liver of E12 *rela*$^{-/-}$ c-*rel*$^{-/-}$ embryos fail to differentiate *in vitro* due to apoptosis (Grossmann *et al.*, 1999). These findings support a model in which the activation of Rel/NF-κB in response to stimuli that promote monocyte differentiation operates via an IKKβ-independent pathway.

Transgenic mice

Rel/NF-κB transgenic mice

Despite the rearrangement and amplification of various Rel/NF-κB and IκB genes in human leukemias and lymphomas (Rayet and Gélinas, 1999), targeted over-expression of RelA (Perez *et al.*, 1995) or RelB (Weih *et al.*, 1996) in the thymocytes of transgenic mice does not lead to the development of thymic or peripheral T-cell abnormalities. However, this conclusion may be somewhat misleading in that over-expression of RelA does not result in an increase in its nuclear levels as there is a corresponding increase in endogenous IκBα, with which RelA forms a cytoplasmic complex. Over-expression of RelB, however, is linked to an increase in nuclear κB site binding activity (Weih *et al.*, 1996). Taken together, these findings indicate that IκBα differentially regulates RelA and RelB in thymocytes.

Transgenic mice over-expressing the v-Rel oncoprotein (Gilmore, 1999) in thymocytes develop T-cell leukemias (Carrasco *et al.*, 1996). The major nuclear v-Rel-containing DNA-binding complexes expressed in these tumors are v-Rel homodimers and v-Rel/p50 heterodimers. v-Rel-induced tumors develop faster in mice homozygous for the null allele of *nfkb1*, suggesting p50 retards v-Rel-mediated leukemogenesis (Carrasco *et al.*, 1996). However, over-expression of IκBα in v-Rel transgenic mice (Carrasco *et al.*, 1997), which selectively reduces the nuclear expression of v-Rel/p50 heterodimers but not v-Rel homodimers in thymocytes, retards leukemia onset and changes the characteristics of the disease. This suggests that the different v-Rel-containing dimers may play distinct roles in T-cell leukemogenesis.

IκB transgenic mice

Over-expression of IκBα$_m$ and IκBβ$_m$ Mutant IκBα and IκBβ proteins (IκBα$_m$ and IκBβ$_m$) that are no longer susceptible to signal-induced degradation have been expressed as transgenes to ablate the nuclear expression of Rel/NF-κB complexes in a developmental and tissue-specific fashion. Several groups have independently targeted these IκB 'super repressors' to the T-cell lineage using various T cell-specific

promoters (Attar *et al*., 1998; Boothby *et al*., 1997; Hettmann *et al*., 1999). While thymocyte development proceeds normally in the absence of individual Rel/NF-κB family members, there is a significant reduction in the number of peripheral $CD8^+$ T cells in mice expressing either $I\kappa B\alpha_m$ or $I\kappa B\beta_m$. The proliferative response of those remaining peripheral T cells or thymocytes to various mitogens, including cross-linking to the T-cell receptor, is impaired (Boothby *et al*., 1997; Hettmann *et al*., 1999), and $I\kappa B\beta_m$ transgenics display impaired T cell-dependent immune responses (Attar *et al*., 1997). Cross-linking of CD3 on double-positive (DP) thymocytes ($CD4^+CD8^+$) normally induces apoptosis, however DP thymocytes from $I\kappa B\alpha_m$ transgenic mice are resistant to this form of cell death (Hettmann *et al*., 1999). This suggests that Rel/NF-κB transcription complexes may promote thymocyte cell death under certain circumstances, a finding not inconsistent with the emerging model that these transcription factors can both promote and inhibit apoptosis (see Barkett and Gilmore, 1999).

In stratified epithelium, the mitotically active basal cells cease to divide and undergo terminal differentiation upon outward migration. The observation that NF-κB proteins are cytoplasmic in mitotically active basal cells but localize to the nucleus of differentiated supradermal cells suggested that NF-κB may be involved in the switch from proliferation to growth arrest and differentiation. Consistent with such as model, targeted expression of $I\kappa B\alpha_m$ to the epidermis of transgenic mice leads to epithelial hyperplasia (Setiz *et al*., 1998), while expression of transgenes for constitutively nuclear p50 or RelA mutants leads to an inhibition of epithelial cell growth (Seitz *et al*., 1998). These findings are consistent with the recent observation that IKKα-deficient mice, which fail to activate Rel/NF-κB in epithelial cells also exhibit a block in epithelial cell differentiation coupled with basal cell hyper-proliferation (Hu *et al*., 1999; Takeda *et al*., 1999).

Over-expression of Bcl-3 *bcl-3*, which is rearranged and over-expressed in chronic lymphocytic leukemia (Rayet and Gélinas, 1999), leads to an increase in p50 homodimer DNA-binding activity when over-expressed in transgenic mice (Caamaño *et al*., 1996). This finding contrasts with the transient over-expression of Bcl-3 in cell lines, where Bcl-3 inhibits DNA binding by p50 homodimers (Franzoso *et al*., 1992). Interestingly, over-expression of Bcl-3 in thymocytes does not induce T-cell leukemias. This indicates either that Bcl-3 is only oncogenic in certain cell lineages, that the stage in the transformation process when deregulated Bcl-3 expression occurs is critical, and/or that Bcl-3 over-expression requires additional mutational events to be oncogenic.

Concluding remarks

The information revealed by the mouse models described herein have provided many insights into the physiological roles of the Rel/NF-κB signal transduction pathway in mammals. Noteworthy examples of important, yet unexpected findings revealed by these mice include that morphogenetic and cytokine signals appear to be transmitted by IKKα- and IKKβ-dependent pathways respectively, that different combinations of Rel/NF-κB proteins are crucial in promoting B-cell differentiation and survival at different stages of development, and that Rel/NF-κB factors appear to play a more important anti-apoptotic role in B cells than in T cells. In the future these mice will prove to be crucial reagents for developing new cell models, for examining the roles of Rel/NF-κB in different diseases and for determining which target genes are regulated by particular Rel/NF-κB dimers.

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